Final Scientific Report

Cover Page

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Project Title: Molecular characterization of PBAN G-protein coupled receptors in moth pest species: design of antagonists

Investigators	**Institutions**
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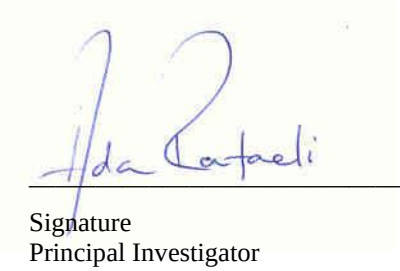
Keywords *not* appearing in the title and in order of importance Avoid abbreviations.

Abbreviations commonly used in the report, in alphabetical order**:**

PBAN-R=Pheromone Biosynthesis Activating Neuropeptide Receptor**;** GPCRs =G-protein coupled receptors

Budget: IS: $**143,000** US: $**142,000** Total: $**285,000**



Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Abstract

Objectives

The proposed research was directed at determining the activation/binding domains and gene regulation of the PBAN-R's thereby providing information for the design and screening of potential PBAN-R-blockers and to indicate possible ways of preventing the process from proceeding to its completion. Our specific aims included: (1) The identification of the PBAN-R binding domain by a combination of: (a) *in silico* modeling studies for identifying specific amino-acid side chains that are likely to be involved in binding PBAN with the receptor and; (b) bioassays to verify the modeling studies using mutant receptors, cell lines and pheromone glands (at tissue and organism levels) against selected, designed compounds to confirm if compounds are agonists or antagonists. (2) The elucidation of the molecular regulation mechanisms of PBAN-R by: (a) age-dependence of gene expression; (b) the effect of hormones and; (c) PBAN-R characterization in male hair-pencil complexes.

Background to the topic

Insects have several closely related G protein-coupled receptors (GPCRs) belonging to the pyrokinin/PBAN family, one with the ligand pheromone biosynthesis activating neuropeptide or pyrokinin-2 and another with diapause hormone or pyrokinin-1 as a ligand. We were unable to identify the diapause hormone receptor from *Helicoverpa zea* despite considerable effort. A third, related receptor is activated by a product of the *capa* gene, periviscerokinins. The pyrokinin/PBAN family of GPCRs and their ligands has been identified in various insects, such as *Drosophila*, several moth species, mosquitoes, *Tribolium castaneum*, *Apis mellifera*, *Nasonia vitripennis*, and *Acyrthosiphon pisum.* Physiological functions of pyrokinin peptides include muscle contraction, whereas PBAN regulates pheromone production in moths plus other functions indicating the pleiotropic nature of these ligands. Based on the alignment of annotated genomic sequences, the primary and secondary structures of the pyrokinin/PBAN family of receptors have similarity with the corresponding structures of the capa or periviscerokinin receptors of insects and the neuromedin U receptors found in vertebrates.

Major conclusions, solutions, achievements

Evolutionary trace analysis of receptor extracellular domains exhibited several class-specific amino acid residues, which could indicate putative domains for activation of these receptors by ligand recognition and binding. Through site-directed point mutations, the 3rd extracellular domain of PBAN-R was shown to be critical for ligand selection. We identified three receptors that belong to the PBAN family of GPCRs and a partial sequence for the periviscerokinin receptor from the European corn borer, *Ostrinia nubilalis*. Functional expression studies confirmed that only the C-variant of the PBAN-R is active. We identified a non-peptide agonist that will activate the PBAN-receptor from *H. zea*. We determined that there is transcriptional control of the PBAN-R in two moth species during the development of the pupa to adult, and we demonstrated that this transcriptional regulation is independent of juvenile hormone biosynthesis. This transcriptional control also occurs in male hair-pencil gland complexes of both moth species indicating a regulatory role for PBAN in males. Ultimate confirmation for PBAN's function in the male tissue was revealed through knockdown of the PBAN-R using RNAi-mediated gene-silencing.

Implications, both scientific and agricultural

The identification of a non-peptide agonist can be exploited in the future for the design of additional compounds that will activate the receptor and to elucidate the binding properties of this receptor. The increase in expression levels of the PBAN-R transcript was delineated to occur at a critical period of 5 hours post-eclosion and its regulation can now be studied. The mysterious role of PBAN in the males was elucidated by using a combination of physiological, biochemical and molecular genetics techniques.

Research Achievements

In silico modeling studies: Refining the model of the GPCR-ligand complex

In our study, evolutionary trace analysis on the identified insect receptor sequences was conducted to predict the putative ligand recognition and binding sites of the pyrokinin/PBAN family of receptors. The evolutionary trace analysis of four class-specific receptors indicated several amino acid residues that are conserved in the transmembrane domains that are embedded in the cell membrane. The receptor extracellular domains exhibit several class-specific amino acid residues, which could indicate putative domains for activation of these receptors by ligand recognition and binding.

Mutant receptors and design of PBAN-antagonists

Previously we characterized the PBAN receptor (PBAN-R) active binding domains using chimeric GPCRs and proposed that extracellular loop 3 is critical for ligand selection. Here, we characterized the 3rd extracellular domain of PBAN-R through site-directed point mutations. Results are discussed in context of the structural features required for receptor activation using receptor activation experiments and *in silico* computational modeling. Following the above published results, a site-directed mutagenesis kit was utilized to make three point mutations substituting alanine in place of the normal amino acid. The alanine substituted mutants were placed into a vector and expressed in Sf9 cells to analyze for functionality. However, we experienced a very low expression level of the mutants and were unable to determine functionality using our calcium assay and fluorescent microscope. In addition the microscope and software were changed and we were unable to utilize the new configuration in our assays. Therefore we were unable to complete this portion of the objectives.

We were successful in identifying three receptors that belong to the PBAN family of GPCRs from the European corn borer, *Ostrinia nubilalis*. We also identified a partial sequence for the periviscerokinin receptor. Quantitative PCR of mRNA for all three receptors indicates differential expression in various life stages and tissues. All three splice variants of the PBAN-R were identified and all variants were found in pheromone gland tissue. Immunocytochemical identification of expressed receptors indicates that all three variants and the diapause hormone receptor can be expressed to the cell surface of Sf9 cells. The A- and B-variants were not active in our functional assay, which confirms studies from other moths.

Functional expression of the C-variant indicates that it is has a 44 nM half effective concentration for activation by PBAN. The diapause hormone-receptor was activated by diapause hormone with a 150 nM half effective concentration.

Design of antagonists

Preliminary evidence has identified a non-peptide agonist (NPA) that will activate the PBAN-receptor from *H. zea*. This finding is important because it indicates that a non-peptide small molecule can activate the receptor. The NPA structure can now be used to design additional compounds that will activate the receptor. In addition, modelling of the NPA bound to the PBAN-receptor could provide information about the design of an antagonist.

Treatment	Pheromone amount, ng ± SEM
Control	10.3 ± 4.2
PBAN 20 pmol	98.1 ± 16.6
NPA-1 100 pmol	88.0 ± 11.0

Elucidation of the molecular regulation mechanisms of the PBAN-R: Developmental influences

We aimed at elucidating the temporal differential pattern of the PBAN-R gene expression levels and the role of Juvenile Hormone in adults of *Helicoverpa armigera*. The differential expression of the PBAN-R in *H. armigera* was compared to the silkworm, *Bombyx mori*, thereby comparing representative species that both employ different reproductive strategies and also express different PBAN-receptor types. Three hour post-eclosion *H. armigera* females revealed low PBAN-R transcript levels and females at this time were unable to produce sex pheromone in response to PBAN. The increase in expression levels of the PBAN-R transcript was delineated to occur at a critical period of 5 hours post-eclosion. Using time points which correspond to the earlier maturation in *B. mori*, up-regulation of the PBAN-R occurs 1 day prior to eclosion increasing 1-2 days post-eclosion, but the precise timing was not delineated in this species. Thus, by demonstrating an age-dependent pattern of expression we allude to the fact that transcriptional regulation indeed must occur. We herein demonstrate that PBAN-R expression levels increase normally when females are decapitated or head-ligated, removing the source of JH, before peak transcript levels are reached.

Similarly, sex pheromone production can be induced by PBAN in such decapitated females. Thus, the present study contests the hypothesis that the PBAN-R gene transcription is under the control of JH as was previously implied.

PBAN-R of male aedeagus complexes

We showed the presence of the PBAN-R in the aedeagus of *H. armigera* males. Moreover, we showed that both the male and the female transcripts undergo transcriptional regulation, and that it is not specific to sex pheromone production in the female pheromone gland. These findings confirm the presence of an up-regulated PBAN-R in male aedeagi of two moth species thereby emphasizes the true functionality of this gene in the males. Results showed that PBAN and its receptor participate in the regulation of free fatty acids and derived components in male complexes: myristic, palmitic, stearic and oleic acids and alcohol components: hexadecanol, cis-11 hexadecanol and octadecanol. Furthermore, hexane extracts of male complexes revealed that component levels are regulated photoperiodically showing a diel periodicity. The significant photoperiodic increases in hexadecanol, cis-11 hexadecanol and octadecanol components reveal that these alcohols are the major volatile components in this tissue. Since this response is coincident with female pheromone production, which is regulated by PBAN-PBAN-R binding, further studies were designed to examine the effect of synthetic *Hez*PBAN. A significant stimulation of component levels occurred as a result of the addition of synthetic *Hez*PBAN to decapitated males. Ultimate confirmation for PBAN's function in this tissue was revealed through knockdown of the PBAN-R using RNAi-mediated gene-silencing. Injections of PBAN-R dsRNA into the male hemocoel significantly inhibited levels of the various male components by 58-74%.

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List of publications

Reviewed Journal Articles:

1. Rafaeli, A. (2009). Pheromone Biosynthesis Activating Neuropeptide (PBAN): Regulatory Role and Mode of Action. *General and Comparative Endocrinology* 162: 69–78.
2. Bober, R., and Rafaeli, A. (2009). Pheromone Biosynthesis Activating Neuropeptide (PBAN) and its G-protein coupled receptor. In: "*Short views on Insect Molecular Biology*" (eds. M. Krishnan and K. Chandrasekar) pp.131-145.
3. Bober, R., Azrielli, A. and Rafaeli, A. (2010). Developmental regulation of the Pheromone Biosynthesis Activating Neuropeptide-Receptor (PBAN-R): Re-evaluating the role of Juvenile Hormone. *Insect Molecular Biology* 19, 77–86.
4. Choi, M-Y., and Jurenka, R.A. (2010). Site-directed mutagenesis and PBAN activation of the *Helicoverpa zea* PBAN-receptor. *FEBS Letters* 584, 1212–1216.
5. Bober, R., and Rafaeli, A. (2010). Gene-silencing reveals the functional significance of Pheromone Biosynthesis Activating Neuropeptide Receptor (PBAN-R) in a male moth. *Proceedings of National Academy of Sciences, USA 107, 16858-16862.*
6. Jurenka, R., and Nusawardani, T. (2011).The pyrokinin/pheromone biosynthesis-activating neuropeptide (PBAN) family of peptides and their receptors in Insecta: evolutionary trace indicates potential receptor ligand-binding domains. *Insect Molecular Biology* 20, 323-334.
7. Jurenka, R. and Rafaeli, A. (2011) Regulatory role of PBAN in sex pheromone biosynthesis of heliothine moths. *Frontiers in Endocrinology* 2, 46.

Conference Abstracts:

1. Rafaeli, A. (2008). Pheromone Biosynthesis Activating Neuropeptide (PBAN) and its G-protein Coupled Receptor. Lecture presented at the 24th Conference of the European Comparative Endocrinologists, Genoa, Italy, 2-6 September.
2. Jurenka, R.A. (2008). Peptides and Receptors in Insect Physiology. American Chemical Society Kansas City, Spencer Award recognition of David Schooley.
3. Jurenka, R.A. (2008). Peptides, Receptors, and Pheromone Biosynthesis" Entomological Society of America, Reno, Nevada.
4. Jurenka, RA., Rafaeli, A., Stern, P. (2009**).** The Pyrokinin/PBAN-receptor Family of GPCRs and Peptide Ligands in Arthropoda: Comparison with Neuromedin U in Vertebrates. Poster presented at the 3rd International Arthropod Genomics Symposium: Frontiers in Arthropod Genomics, Kansas City, MO, 11 -14 June.
5. Jurenka, R., Rafaeli, A., Stern, P. (2009). The Pyrokinin/PBAN-receptor Family of GPCRs and Peptide Ligands in Arthropoda: Comparison with Neuromedin U in Vertebrates. Arthropod Genomics meeting. Kansas City, MO, June.
6. Jurenka, R., Rafaeli, A., Stern, P. (2009). The Pyrokinin/PBAN-receptor Family of GPCRs and Peptide Ligands in Arthropoda: Comparison with Neuromedin U in Vertebrates. ESA national meeting. Indianapolis, Dec.
7. Bober, R. and Rafaeli, A. (2009). Developmental regulation of the Pheromone Biosynthesis Activating Neuropeptide-Receptor (PBAN-R): Re-evaluating the role of Juvenile Hormone. Poster presented at the 25th Annual Meeting of the International Society of Chemical Ecology, Neûchatel, Switzerland, 23-27 August.
8. Rafaeli, A. (2010). Molecular mechanisms underlying sex-pheromone communication in Lepidoptera. Genomics of insect pests to agriculture held in Rehovot, Israel, 24-25 May 2010.

9. Rafaeli, A. (2010). The PK/PBAN family of insect neuropeptides. IXth European Congress of Entomology, Budapest, Hungary, 22-27 August.
10. Bober, R. and Rafaeli, A. (2010). Developmental regulation of the Pheromone Biosynthesis Activating Neuropeptide Receptor (PBAN-R). 25th Conference of the European Comparative Endocrinologists, Pecs, Hungary 31 August– 4 September.
11. Jurenka, R.A. and Nusawardani, T. (2010).The pyrokinin/PBAN family of GPCRs: evolutionary trace analysis indicates putative ligand recognition and binding sites. 4th Annual Arthropod Genomics Symposium. Arthropod Genomics: New Approaches and Outcomes. Kansas City, Missouri, June.
12. Jurenka, R.A. (2010). Pheromone biosynthesis in moths. EC Advanced Workshop on Infochemical Communication Technology, Granada, Spain.
13. Rafaeli, A. (2012). RNAi technology reveals the roles of G-protein coupled receptors in moth reproductive behavior. International Congress of Entomology (ICE), Daegu, South Korea, August 19-25.

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged		2	3	5
Submitted, in review, in preparation	1			1
Invited review papers	1		1	2
Book chapters				
Books				
Master theses				
Ph.D. theses			1	
Abstracts	3	4	6	13
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

1. Rachel Bober ARO - ID # 03351300-3
2. Tyasning Nusawardani - USA

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1		
Longer Visits (Sabbaticals)				

Description Cooperation:

The research teams in the ARO and Iowa involved in the present proposal have been addressing the biochemical mode of action of pheromonotropic stimulation in moths in the quest at understanding fully the receptor-ligand interactions at the cellular level and have benefited from previous collaborations on the subject. The synthesis of the ARO and Iowa research teams facilitated rapid advances in this field and exchange of knowledge using complementary expertise of both laboratories in biochemistry and physiology of moth species. An exchange trip to the USA by the Israel PI aided in the strengthening of this collaboration.